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Supramolecular Sensor for Cancer-Associated Nitrosamines

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Supporting Information

ABSTRACT: A supramolecular assay based on two fluorescent cucurbit[*n*]uril probes enables the recognition and quantification of nitrosamines, including cancer-associated nitrosamines, compounds that are difficult to recognize. The cross-reactive sensor leverages weak interactions and competition among the probe, metal, and guest, yielding high information density in the signal output (variance) and enabling the recognition of structurally similar guests.

N-nitrosamines are ubiquitous yet toxic compounds and potent carcinogens, primarily because of their ability to alkylate DNA.[1] Decarboxylation of amino acids by bacterial enzymes[2] followed by heat processing of the resulting amines generates secondary amines such as pyrrolidine and piperidine.[3] These amines, which are found in foods and meats, form carcinogenic nitrosamines when processed by salting, curing, or smoking or preserved with common food additives such as sodium and potassium nitrite (E249 and E250)[4] to prevent botulism. Also, nitrate additives (E251, E252)[4] give rise to nitrites via bacterial reduction.[2] Finally, some carcinogenic nitrosamines are formed as byproducts of thermal treatment of endogenous chemicals. Typical examples are tobacco-specific *N*-nitrosamines (TSNAs) such as *N*-nitrosonornicotine (NNN) and (4-methylnitrosamino)-1-(3-pyridyl)-1-butanone (NNK) (Figure 1), which play an important role in tobacco-induced carcinogenesis.[1b]

Cucurbit[*n*]urils (CB[*n*]s) form stable complexes with ammonium ions in water.[5] What was not known, however, is that CB[*n*]s and their derivatives can bind nitrosamines, specifically *N*-nitrosodimethylamine (NDMA), *N*-nitrosopiperidine (NPIP), and the TSNAs NNN and NNK (Figure 1). It was surmised that nitrosamines are not sufficiently basic to be protonated above pH 3, and therefore, binding of nitrosamines to CB[*n*]s was considered unlikely.[6] In this study, we found that CBs bind nitrosamines. We presume that the contribution of the hydrogen bonding of the protonated guest may be less prominent and that other interactions (e.g., hydrophobic, dipole−dipole, etc.) come to the fore.

CB[*n*] molecular containers have been used in fluorescent sensors, but their nonchromophoric nature limits their sensing applications to dye displacement assays relying on competitive processes.[7] We previously developed a CB[*n*]-based turn-on fluorescent probe for sensing of diamines[8a] based on fluorescent probe **1**. In this work, the recognition power of this system has been augmented with the abilities of acyclic CB[*n*]-type probe **2**.[8b,c]

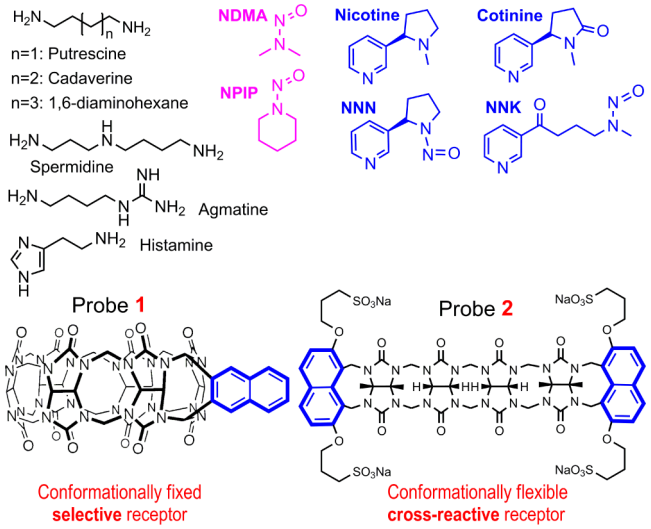

Figure 1. Structures of guests and probes used in this study.

For the design of array sensors, particularly ones with minimum-sized arrays, it is important to include both selective and cross-reactive probes.[9] This concept was used in the selection of probes **1** and **2** for the two-probe sensor described herein. The cyclic structure of CB[6] derivative **1** imparts high selectivity for smaller guests (e.g., NDMA, NPIP). Therefore, we synthesized the cross-reactive[10] probe **2** possessing a more structurally flexible acyclic receptor that is capable of accommodating a more structurally diverse group of guests, including nitrosamines, (−)-nicotine, and (−)-cotinine.

Probes **1** and **2** are fluorescent because of the presence of naphthalene moieties, and this fluorescence is partly quenched by coordination of metal ions to the ureidyl C═O-decorated portals (Figure 2). The Supporting Information (SI) shows quenching isotherms recorded for probes **1** and **2** in the presence of Eu^{3+}, Yb^{3+}, Zn^{2+}, Ba^{2+}, and Hg^{2+}. The two probes

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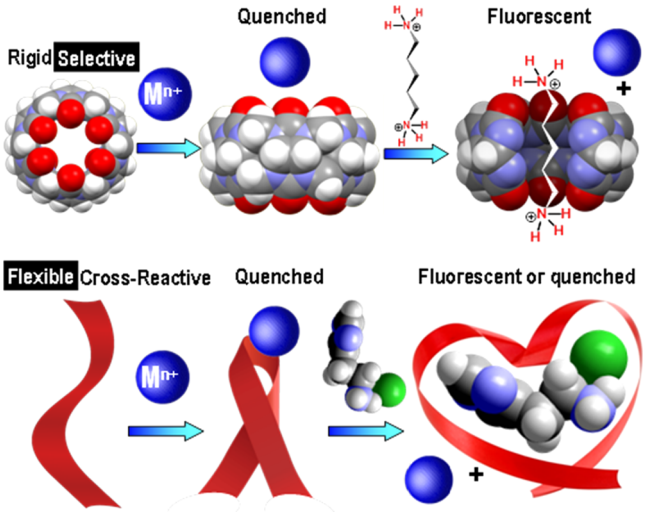

Figure 2. Probes **1** and **2** are fluorescent, and their fluorescence is partly quenched by the bound metal ion. Depending on the guest structure, the interaction between the probe and the guest results in either recovery or further quenching of the fluorescence. Probes **1** and **2** utilize complementary features of selective probe **1**, which has a narrow cavity displaying a preference for aliphatic guests, and flexible probe **2**, which adapts its size to a wider variety of guests.

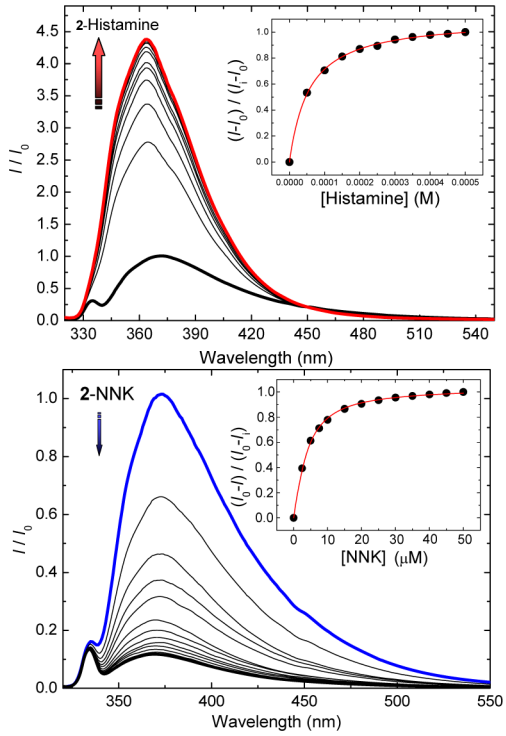

Figure 3. Top: Fluorescence spectra of **2** (3 μM) with Eu^{3+} (300 μM) upon the addition of histamine in water at pH 3. λ_{ex} = 301 nm. Bottom: Spectra of **2** (3 μM) upon the addition of NNK in water at pH 3. λ_{ex} = 301 nm.

have different affinities for metal ions. Probe **1** exhibited K_a values (M^{-1}) of 6.2 × 10^3, 2.1 × 10^3, and 2.6 × 10^2 for Eu^{3+}, Yb^{3+}, and Zn^{2+}, respectively; the addition of Ba^{2+} and Hg^{2+} did not yield appreciable changes in the fluorescence. In contrast, probe **2** exhibited the following K_a values (M^{-1}): Eu^{3+}, 1.1 × 10^4; Yb^{3+}, 1.3 × 10^4; Ba^{2+}, 4.7 × 10^3; Hg^{2+}, 8.0 × 10^2; Zn^{2+}, <50.

In the presence of a competitive guest, the metal ion is displaced, and the fluorescence is either recovered or reset to a different level depending on the affinity of the guest and its ability to impact the fluorescence of the probe. For example, amines such as histamine regenerate the fluorescence (Figure 3 top), while pyridine-containing amines such as nicotine and cotinine quench the probe fluorescence. The magnitude of the change in the fluorescence intensity is a key for generating a signal with high information density suitable for the development of array sensors with high ability to differentiate between structurally similar guests (Figure 3).

The present supramolecular sensor utilizes several unique features that affect the fluorescence response. The first is the complementary behavior of selective probe **1**, with a narrow cavity, and flexible probe **2**, accommodating a wider variety of guests (cf. cross-reactivity) (Figure 2). Second, the probes show different affinities and responses to different metal ions. Third, probes **1** and **2** display different affinities for the guests, which more or less effectively compete with the quenching metal. For example, probe **1** yielded the following K_a values (M^{-1}) for nitrosamines: NPIP, 1.9 × 10^3; NNN, 2.7 × 10^4; NNK, 4.0 × 10^3; NDMA, 1.2 × 10^4. In contrast, probe **2** exhibited K_a values (M^{-1}) of 5.8 × 10^3 for NPIP, 1.3 × 10^5 for NNN, and 4.1 × 10^5 for NNK but did not show appreciable affinity for NDMA. The apparent affinity constants calculated for each probe–metal–guest combination are listed in the SI. The cumulative effect of the three factors listed above results in unique responses of probes **1** and **2** to various guests. These responses were recorded as fluorescence intensities at 320 and 370 nm from probe–metal–guest solutions using conventional 1536-well plates (see the SI for a detailed description). The response data sets were acquired in the form of a guest (X) × variable (Y) (probe, metal, emission wavelength) matrix, each field being associated with unique fluorescence intensity. Pattern recognition protocols were then used to reveal the guest-specific trends in the response.[9c]

In the qualitative assay, the linear diamines (1,6-diaminohexane, putrescine, cadaverine, agmatine, and spermidine) were used at 12 μM, while histamine, nicotine, cotinine, NDMA, NPIP, NNN, and NNK were used at 50 μM. The sensor responses were analyzed and evaluated by linear discriminant analysis (LDA), a standard tool of statistical multivariate analysis.[11] A preliminary analysis performed using only the data from metal-free and Eu^{3+}-containing solutions suggested excellent recognition capability of the sensor, as illustrated by the 100% correct classification of all 260 data points (12 guests + control, 20 repetitions per guest) using the leave-one-out procedure.

Figure 4 shows the response space defined by the first three canonical factors (F1–F3). The sensor array recognized the guests and sorted them into three groups: amines, nitrosamines, and the tobacco alkaloids nicotine and cotinine. Interestingly, the response to nitrosamines placed these guests between the tobacco alkaloids and the aliphatic amines. The success of the qualitative analysis in recognizing 12 guests, some of them structurally very similar, validated the strategy based on leveraging of selectivity and cross-reactivity in the two-probe sensor.

This positive outcome enabled a semiquantitative assay that used the same array sensor to identify various guest concentrations. The analysis of the carcinogenic nitrosamines NNN and NNK is shown in Figure 5. These results show a clear dependence of the fluorescence response on the

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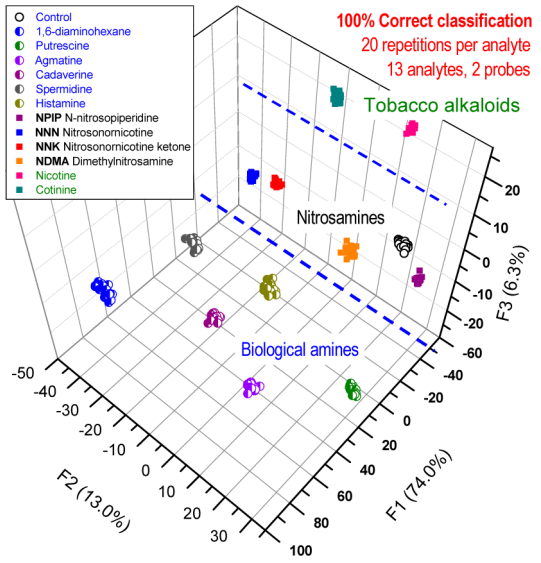

Figure 4. Results of the qualitative LDA of biological amines, cancer-associated nitrosamines, and tobacco alkaloids using an array sensor based on probes **1** and **2**.

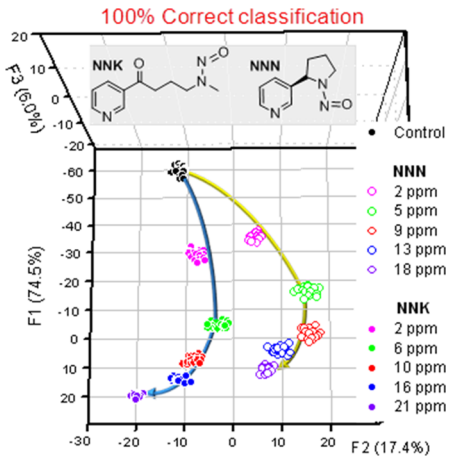

Figure 5. Results of LDA of the semiquantitative assay of NNN and NNK.

concentrations of NNN and NNK suggesting that the array should allow for a rigorous quantitative determination. The sensor successfully quantified NNN/NNK mixtures in the presence of an order-of-magnitude excess of nicotine (50 μM). Both NNN and NNK are nicotine transformation products, and nicotine may be found in the solution of the TSNA guests. Inspection of the binding affinities (listed in the SI) suggested that whereas NNN and NNK display higher affinities for probes **1** and **2**, nicotine is a competing interferent. Because probe **2** displays higher affinity than probe **1** for NNN/NNK relative to nicotine, we decided to use only the response data recorded using probe **2**. While using this abbreviated data set did not take advantage of all the information available, it reduced the time and effort required for the analysis.

For the quantitative analysis of NNN/NNK mixtures, we used a support vector machine (SVM) regression method, which is more suitable for modeling complex responses and nonlinear behavior of the data.[12] The SVM regression was successful and allowed for simultaneous prediction of multiple guest concentrations even in the presence of excess nicotine. In fact, this method allowed the use of an abbreviated data set comprising only data obtained with probe **2** Eu^{3+}, Yb^{3+}, and Ba^{2+}, thereby limiting the amount of work required. Here, we used five guest concentrations to model the behavior of the data and two different guest concentrations to validate the model simultaneously. It was possible to evaluate the model by visual inspection of the plots of predicted versus actual concentration for NNN and NNK (Figure 6), attesting to the predictive power of the model.

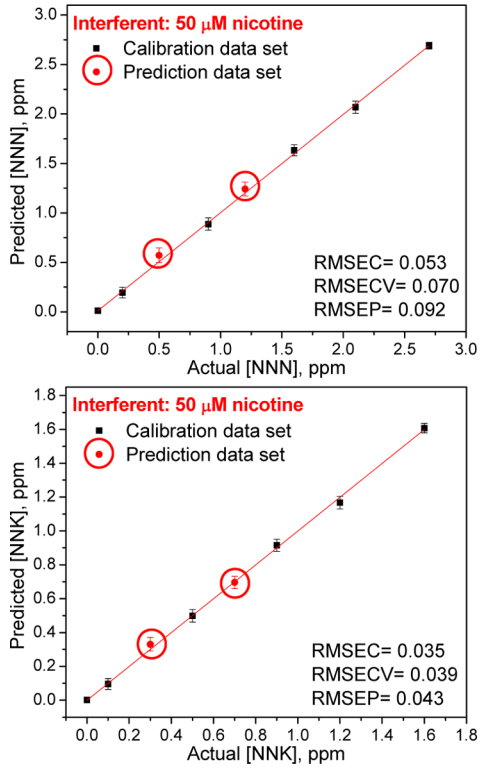

Figure 6. Results of the SVM regression for quantitative analysis of (top) NNN and (bottom) NNK mixtures in the presence of interfering nicotine (50 μM). The plots of actual vs predicted concentration show high accuracy of prediction for multiple guest concentrations. The values of the root-mean-square errors (RMSEs) of calibration (C), cross-validation (CV), and prediction (P) (shown as insets) attest to the high quality of the model and prediction.

Finally, we established the limit of detection (LOD)[13] for several guest analytes of interest, including histamine (0−7 ppm; LOD = 0.09 ppm), nicotine (0−12 ppm; LOD = 0.75 ppm), NNN (0−18 ppm; LOD = 0.05 ppm), and NNK (0−21 ppm; LOD = 0.27 ppm). In general, the LOD values are comparable to or lower than the requirements of current methods used in food safety applications,[14a] which rely chiefly on solid-phase extraction/GC−MS or the current EPA 521 method.[14b] The LODs for nitrosamines in various foods are 0.5−0.9 ppm using GC−MS.

In summary, we have demonstrated the first supramolecular assay for cancer-associated nitrosamines. This simple cross-reactive array sensor utilizes two cucurbit[*n*]uril-type probes displaying complementary selectivities, thereby imparting the ability to recognize biologically active amines and nitrosamines. Fluorimetric titrations of the individual probes showed highly variable guest-dependent changes in fluorescence. The assay requires only simple laboratory instrumentation yet displays an

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excellent recognition profile for a large number of guests (two probes recognized 13 guests) in a qualitative as well as quantitative manner. Quantitative analysis successfully determined the concentrations of individual components in mixtures of the tobacco-specific *N*-nitrosamines NNN and NNK, even in the presence of an order-of-magnitude higher concentration of nicotine interferent. The successful analysis of nitrosamines was particularly unexpected because unlike amines, the less basic nitrosamines do not easily form hydrogen-bonded bridges to the CB[*n*] carbonyl moieties. We believe that this study opens up new avenues for the application of CB[*n*]-type receptors in sensing and could aid in the development of simple sensors for amines and nitrosamines in the future.

■ ASSOCIATED CONTENT

Supporting Information

Synthesis and characterization of probe **2**, fluorescence spectra, table of K_a values, experimental details for sensing, and data matrices. This material is available free of charge via the Internet at http://pubs.acs.org.

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Notes

The authors declare no competing financial interest.

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Maria E. Kozelkova has been added as a contributing author. The revised version was re-posted on December 12, 2012.